Supporting information

Functional diversity between HSP70 paralogs due to variable interactions with specific co-chaperones.

Despina Serlidaki[1], Maria A. W. H. van Waarde[1], Lukas Rohland[2], Anne S. Wentink[2], Suzanne L. Dekker[1], Maarten J. Kamphuis[1], Jeffrey M. Boertien[1], Jeanette F. Brunsting[1], Nadinath B. Nillegoda[2,3], Bernd Bukau[2], Matthias P. Mayer[2], Harm H. Kampinga[1*] & Steven Bergink[1*]

Fig S1

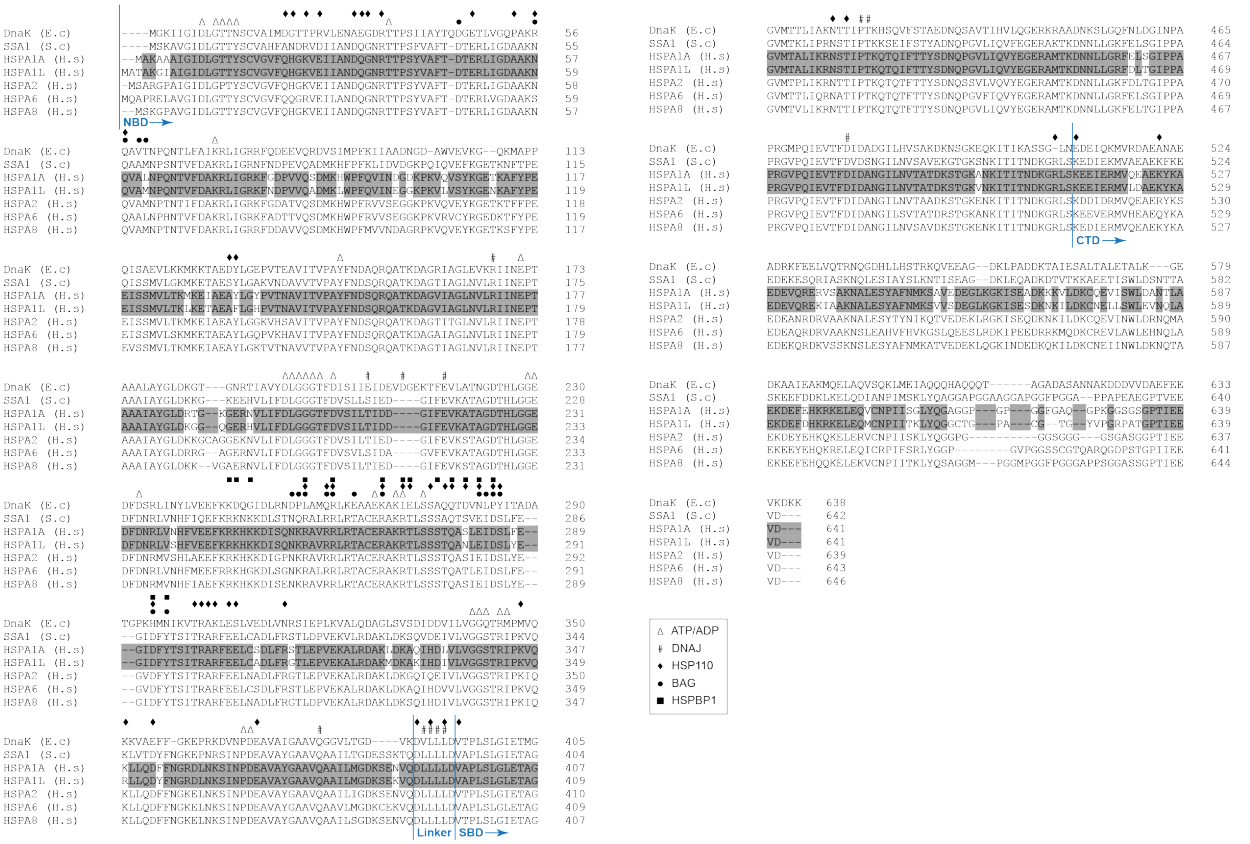


Figure S1: Hsp70 sequence alignment.

Clustal omega sequence alignment of the main cytosolic/nuclear Hsp70s of E.coli (DnaK), S. cerevisiae (Ssa1) and H. sapiens (HSPA1A, HSPA1L, HSPA2, HSPA6, HSPA8). Identical residues between HSPA1A and HSPA1L are shown in grey. Symbols indicate the predicted interaction sites with ATP/ADP and co-chaperones, based on previously published data from various Hsp70 and co-chaperone homologues: (Δ) ATP/ADP (34, 71–76), (#) DNAJ (9), (♦) Hsp110 (53, 54), (●) BAG (55, 77, 78) and (■) HSPBP1 (56). Sequence identity percentage between the Hsp70 family members is shown in table S1.

Fig.S2

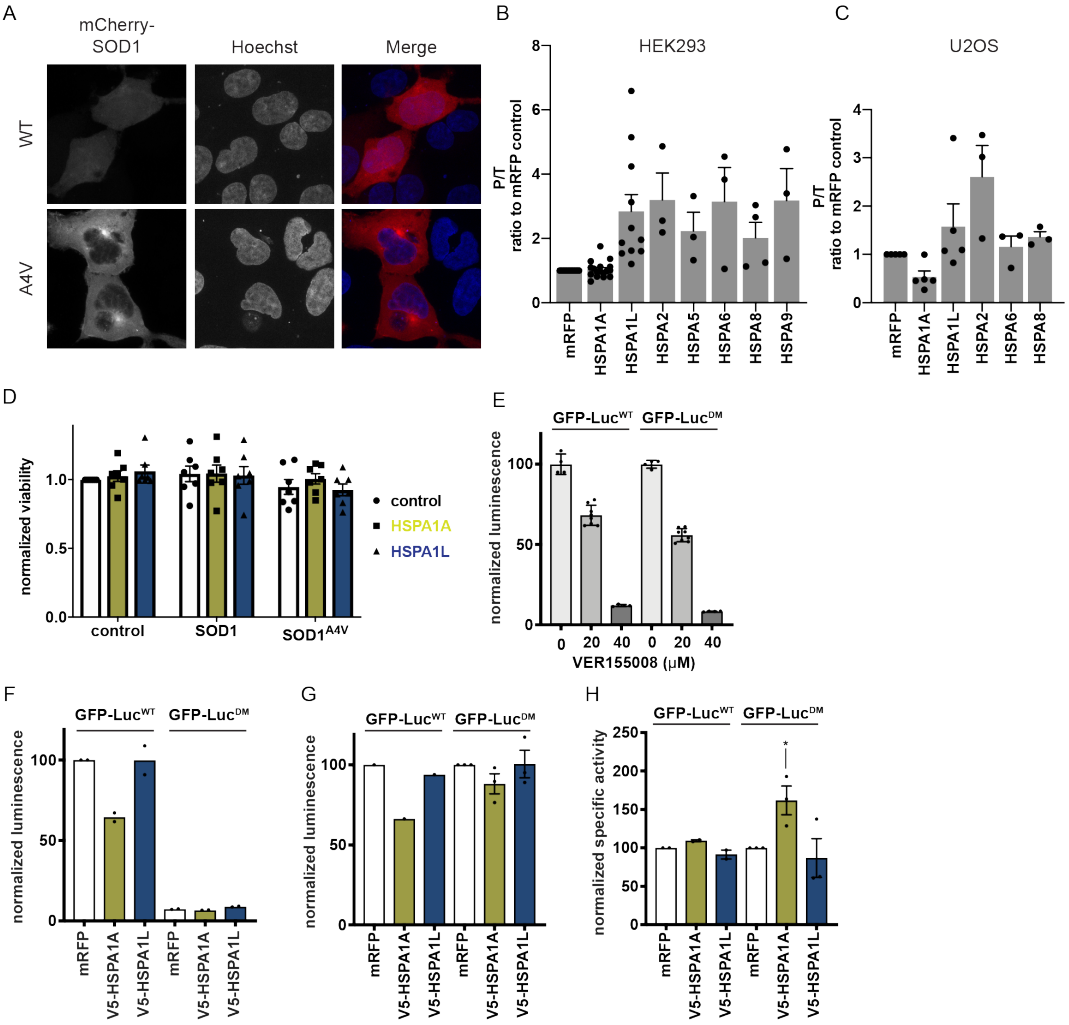


Figure S2: SOD1^{A4V} aggregation and Luciferase^{DM} activity. A-D: SOD1^{A4V} aggregation. **A** mCherry-SOD1^{A4V} forms aggregates in HEK293 cells after 48 hours. Fluorescence microscopy images of mCherry-SOD1^{WT} and mCherry-SOD1^{A4V} (left column) and Hoechst (middle column) and the merge (right column, mCherry in red, DNA in blue) are shown. Scale bar 10μm. **B-C** Graphs of the Hsp70 screens shown in Figures 1D (HEK293) and 1E (U2OS) depicting the pellet (P) fraction of SOD1 as a ratio to the total (T) fraction. **B** here represents HEK293 screen and **C** U2OS, error bars with s.e.m. **D** Viability of HEK293 cells expressing SOD1^{WT} or SOD1^{A4V}. HEK293 cells were transfected for 48 hours with the indicated constructs and viability was measured using an MTS assay. Graphs depicts the normalized colorimetric values. E-H: Luciferase^{DM} activity is dependent on Hsp70. **E** VER155008 inhibits GFP-Luc^{DM} as well as GFP-Luc^{wt} activity. HEK293 cells expressing GFP-Luc^{wt} or GFP-Luc^{DM} were treated with the indicated dose of VER155008 for 24 hours. Luminescence values corrected for protein levels and normalized to the untreated conditions (i.e. to either GFP-Luc^{WT} or GFP-Luc^{DM}) are shown. **F** GFP-Luc^{DM} retains reduced luciferase activity. HEK293 cells were transfected with either GFP-Luc^{WT} or GFP-Luc^{DM} in the presence of mRFP, V5-HSPA1A or V5-HSPA1L. Luminescence activities normalized to mRFP GFP-Luc^{WT} are shown. **G** Total GFP-Luc^{DM} activities are not altered by HSPA1A or HSPA1L. HEK293 cells transfected with GFP-Luc^{wt} or GFP-Luc^{DM} together with either mRFP (control), V5-HSPA1A or V5-HSPA1L. Luminescence values normalized to mRFP are shown. **H** HSPA1A increases the specific activity of GFP-Luc^{DM}. HEK293 cells transfected with GFP-Luc or GFP-Luc^{DM} together with either mRFP (control), HSPA1A or HSPA1L. Luminescence values normalized to mRFP treatment and corrected for GFP-Luc^{WT} or GFP-Luc^{DM} protein expression levels are shown.

Fig.S3

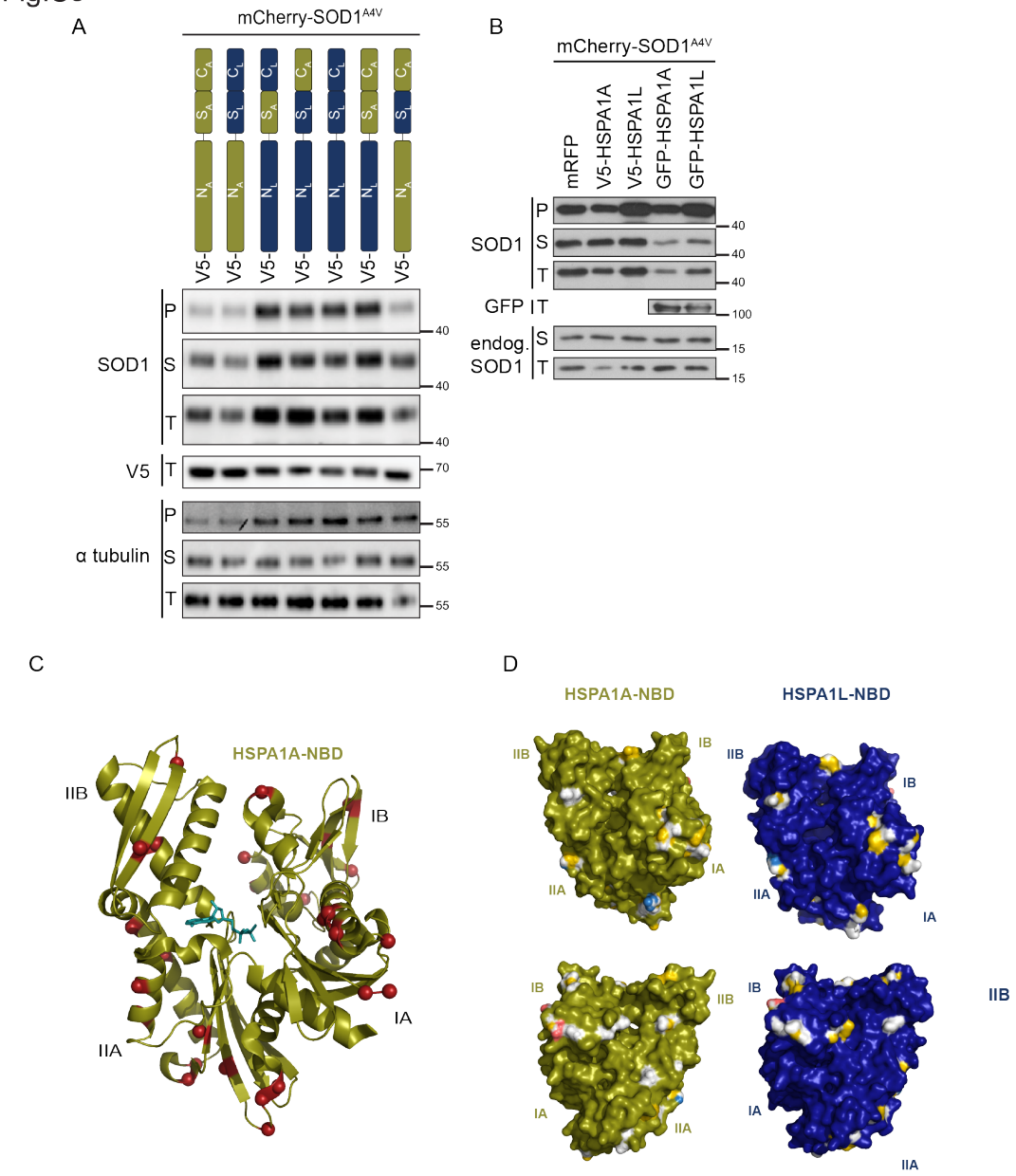


Figure S3: Hsp70 domain and subdomain differences affecting the functionality of HSPA1A or HSPA1L. A Effect of SBD or CTD swapped chimeras between HSPA1A and HSPA1L on SOD1^{A4V} aggregation. Western blot of NP40 fractionation of HEK293 cells co-expressing mCherry-SOD1^{A4V} and V5-tagged chimeric proteins with swapped SBDs or CTDs between HSPA1A (yellow) and HSPA1L (blue) for 48 hours. **B** Effect of V5-versus GFP-tagged Hsp70s. Western blot of NP40 fractionation of HEK293 cells co-expressing mCherry-SOD1^{A4V} and V5- or GFP-tagged HSPA1A and HSPA1L for 48 hours. Endogenous SOD1 is used as a loading control. **C** Positions of the non-conserved residues between HSPA1A and HSPA1L NBDs. Ribbon model of HSPA1A-NBD (PDB ID: 3JXU, (34); yellow) and the positions of the residues that are not conserved in HSPA1L (red spheres). Subdomains of lobes I and II are noted for orientation. ADP is colored in light blue. **D** Interaction surface of HSPA1A or HSPA1L NBDs. Surface representations of HSPA1A-NBD (PDB ID: 3JXU, (34); yellow-green) and HSPA1L-NBD (PDB ID: 3GDQ, (34); blue). For the non-conserved residues, hydrophobic surfaces are colored in yellow, negatively charged in red, positively charged in light blue and the remaining in white (PyMOL script YRB, (79)). Subdomains IA/B and IIA/B are marked for orientation. The lower structures are rotated by 180º as compared to the upper structures.

Fig.S4

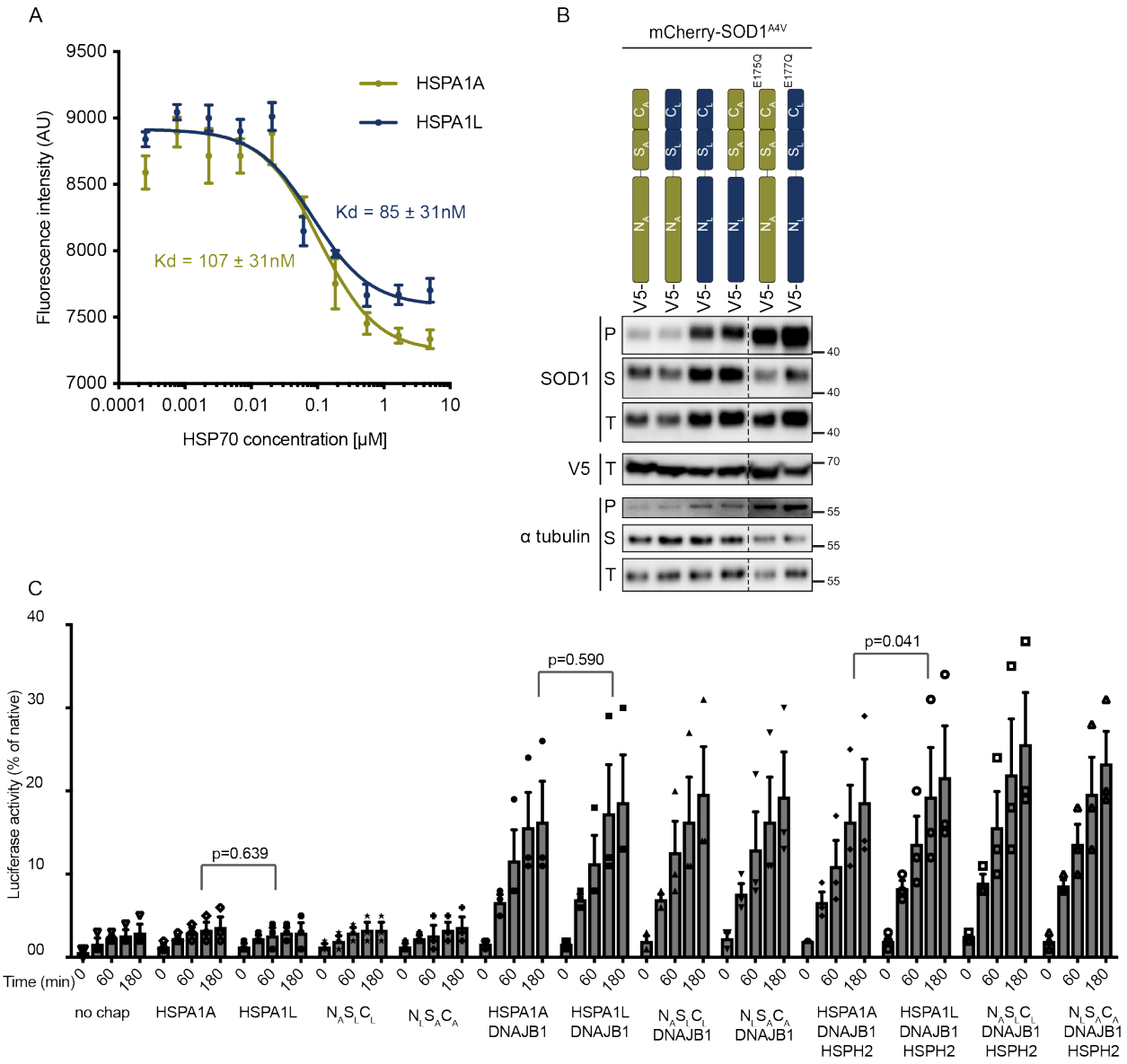
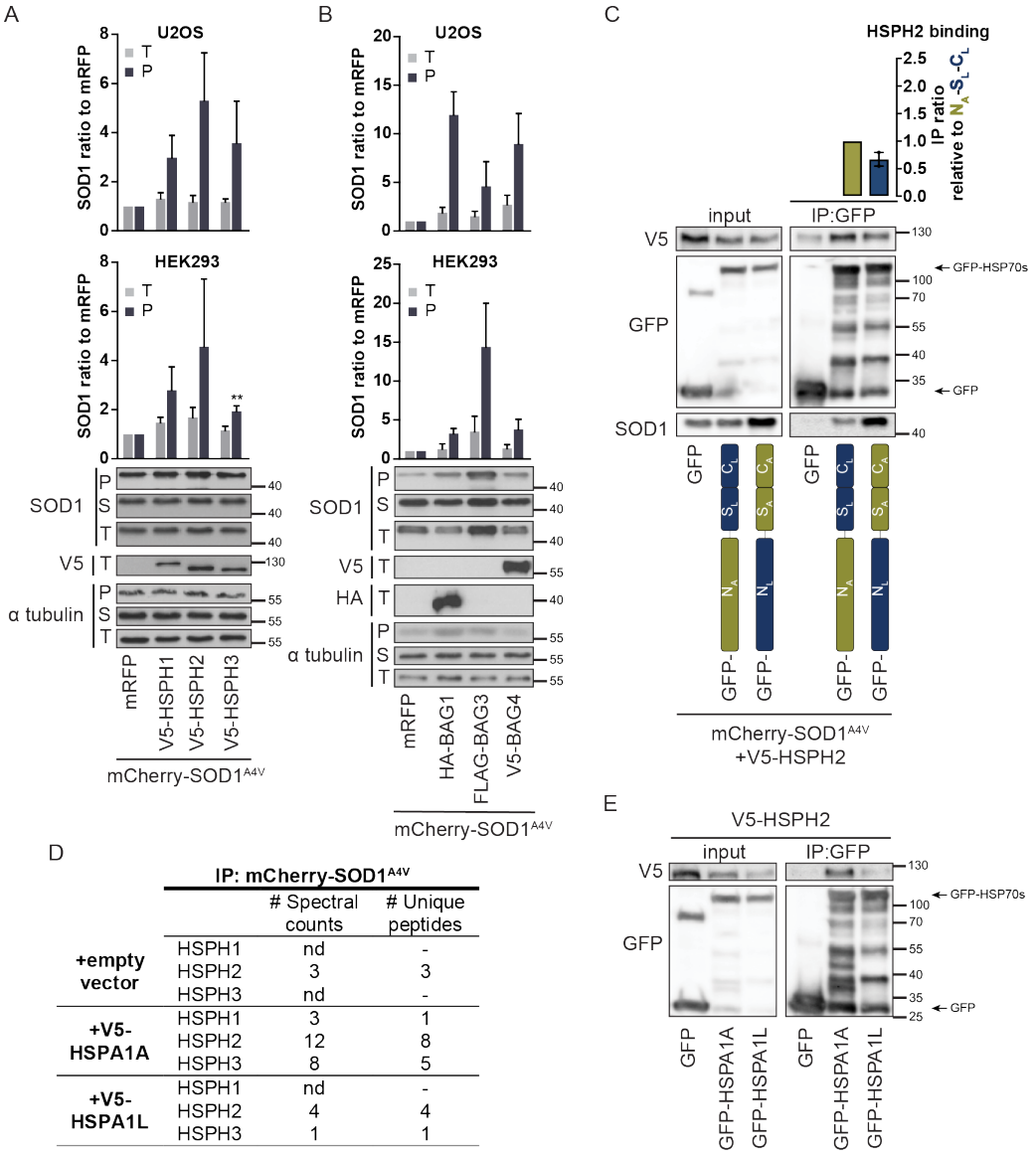


Figure S4: Refolding activity of HSPA1A, HSPA1L and NTD swapped chimeras. A Interaction of a canonical NEF with HSPA1A and HSPA1L. Steady state fluorescence intensity changes in 20nM HSPH2^{FLASH} upon titration of HSPA1A (green) or HSPA1L (blue). Error bars indicate the SEM of three independent experiments. **B** HSPA1A or HSPA1L ATPase-deficient mutants on SOD^{A4V} aggregation. Representative western blots of NP40 fractionation of HEK293 cells co-expressing for 48 hours mCherry-SOD1^{A4V} and V5-tagged HSPA1A (yellow) or HSPA1L (blue) or their ATPase-deficient mutants, HSPA1A^{E175Q} (n=4) and HSPA1L^{E177Q} (n=2) and the NBD swapped chimeras for comparison. **C** Refolding activity of HSPA1A, HSPA1L and chimeras in the presence of DNAJB1 or DNAJB1 and HSPH2. Heat denatured Luciferase was incubated for the indicated time in the presence of the indicated recombinant chaperones. Graph depicts the mean and error bars with s.e.m.. Related-Samples Friedman's Two-Way Analysis of Variance with Post-hoc Mann-Whitney test was performed and p values are indicated.

Fig.S5



D

IP: mCherry-SOD1^{A4V}		# Spectral counts	# Unique peptides
+empty vector	HSPH1	nd	-
	HSPH2	3	3
	HSPH3	nd	-
+V5-HSPA1A	HSPH1	3	1
	HSPH2	12	8
	HSPH3	8	5
+V5-HSPA1L	HSPH1	nd	-
	HSPH2	4	4
	HSPH3	1	1

Figure S5: NEF effect on SOD1^{A4V} aggregation and binding to Hsp70s. A Screen of Hsp110 family members for suppressors of SOD1^{A4V} aggregation. NP40 fractionation of HEK293 (representative western blot and graph) or U2OS (graph only) cells co-expressing mCherry-SOD1^{A4V} and V5-HSPH1-3 for 48 hours. Quantification graphs represent total (T) or NP40-insoluble (P) fraction western blot intensities relative to mRFP control (n=5 for U2OS and n=3-6 for HEK293). **B** Screen of BAG family members for suppressors of SOD1^{A4V} aggregation. NP40 fractionation of HEK293 (representative western blot and graph) or U2OS (graph only) cells co-expressing mCherry-SOD1^{A4V} and HA-BAG1, FLAG-BAG3 or V5-BAG4 for 48 hours. Quantification graphs represent total (T) or NP40-insoluble (P) fraction western blot intensities relative to mRFP control (n=2 for U2OS and n=2 HEK293). In (A&B), error bars with s.e.m., * p=0.01-0.05, ** p=0.001-0.01, *** p<0.001. **C** Interaction of HSPH2 with NBD swaps in the presence of SOD1^{A4V}. Co-expression of GFP-tagged NBD swapped chimeras with V5-HSPH2 and mCherry-SOD1^{A4V} for 48 hours followed by native immunoprecipitation of Hsp70s with GFP nanotrap and western blots using the indicated antibodies. Quantification graph of binding represents ratios of IP intensities of V5/GFP relative to N_A-S_L-C_L chimera measurements (n=2). Error bars with s.e.m. **D** List of Hsp110 interactors of SOD1^{A4V} by mass spectrometry. mCherry-

SOD1^{A4V} was co-expressed with either empty vector, V5-HSPA1A or V5-HSPA1L and soluble mCherry-SOD1^{A4V} was precipitated using RFP nanotrap. Samples were subjected to mass spectrometry analysis and Hsp110 interactors were identified for each sample. Number of spectral counts and unique peptides are noted for each protein. **E** Interaction of HSPH2 with HSPA1A or HSPA1L without the presence of SOD1^{A4V}. Co-expression of GFP-HSPA1A or GFP-HSPA1L with V5-HSPH2 for 48 hours, followed by native immunoprecipitation of Hsp70s with GFP nanotrap and western blots using the indicated antibodies.

Fig S6

Figure S6: Hsp110 sequence alignment.

Clustal omega sequence alignment of Hsp110s of S. cerevisiae (Sse1) and H. sapiens (HSPH1, HSPH2, HSPH3). Symbols (▼) indicate the predicted interaction sites with Hsp70s, based on previously published data using mammalian Hsp70s and Sse1 (53, 54). Sequence identity percentage between the Hsp110 family members is shown on table S2.

Table S1. Percentage* of HSP70 family members sequence identity and their subcellular localization.

HSPA1A	HSPA1L	HSPA2	HSPA5	HSPA6	HSPA8	HSPA9	Ssa1	DnaK		
	88.7	83.9	63.6	81.9	85.6	49.4	74.7	49.1	**HSPA1A**	Cytosol,nucleus
88.7		82.5	63.1	79.7	83.1	49.1	74.1	48.4	**HSPA1L**	Cytosol,nucleus
83.9	82.5		64.1	78.6	87.3	49.9	72.9	48.1	**HSPA2**	Cytosol,nucleus
63.6	63.1	64.1		62.3	65.2	50.5	66.0	51.0	**HSPA5**	ER
81.9	79.7	78.6	62.3		78.0	50.6	71.1	48.5	**HSPA6**	Cytosol,nucleus
85.6	83.1	87.3	65.2	78.0		50.2	75.9	47.9	**HSPA8**	Cytosol,nucleus
49.4	49.1	49.9	50.5	50.6	50.2		50.6	60.3	**HSPA9**	Mitochondria
74.7	74.1	72.9	66.0	71.1	75.9	50.6		49.4	**Ssa1**	*S.cerevisiae*
49.1	48.4	48.1	51.0	48.5	47.9	60.3	49.4		**DnaK**	*E.coli*

*Percentages were calculated using Clustal Omega protein sequence alignment program (EMBL-EBI) (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Table S2. Percentage* of HSP70 family members sequence identity and their subcellular localization.

HSPH1	HSPH2	HSPH3	Sse1		
	64.5	59.1	39.8	**HSPH1**	Cytosol,nucleus
64.5		64.4	39.4	**HSPH2**	Cytosol,nucleus
59.1	64.4		37.4	**HSPH3**	Cytosol,nucleus
39.8	39.4	37.4		**Sse1**	*S.cerevisiae*

*Percentages were calculated using Clustal Omega protein sequence alignment program (EMBL-EBI) (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Table S3: List of chaperones mentioned, their alternative names and uniprot IDs

Family Name	*Chaperone Name*	*Alternative names*	*Uniprot ID*
Hsp70 (HSPA)	HSPA1A	HSP70-1, HSP72, HSPA1, HSX70	P0DMV8
	HSPA1L	HSP70-Hom, Hum70t	P34931
	HSPA2	HSP70-2	P54652
	HSPA5	GRP78, BIP	P11021
	HSPA6	HSP70B'	P17066
	HSPA8	HSC70, HSP73, HSPA10	P11142
	HSPA9	GRP75, HSPA9B, mt-HSP70	P38646
Hsp110 (HSPH)	HSPH1	HSP105, HSP110	Q92598
	HSPH2	HSPA4, APG2	P34932
	HSPH3	HSPA4L, APG1, OSP94	O95757
DNAJ (HSP40, JDP)	DNAJA1	DNAJ2, HDJ2, HSJ2, HSDJ	P31689
	DNAJA2	CPR3, HIRIP4	O60884
	DNAJA3	HCA57, TID1	Q96EY1
	DNAJA4	MSTP104	Q8WW22
	DNAJB1	DNAJ1, HDJ1, HSPF1, HSP40	P25685
	DNAJB2	HSJ1, HSPF3	P25686
	DNAJB3	HCG3	Q8WWF6
	DNAJB4	DNAJW, HLJ1	Q9UDY4
	DNAJB5	HSC40	O75953
	DNAJB6	HSJ2, MRJ, MSJ1, HHDJ1	O75190
	DNAJB7	HSC3	Q7Z6W7
	DNAJB8	-	Q8NHS0
	DNAJB9	ERDJ4, MDG1	Q9UBS3
BAG	BAG1	HAP	Q99933
	BAG3	BIS	O95817
	BAG4	SODD	O95429
HSPBP1	HSPBP1	HSPBP	Q9NZL4
HIP	HIP	ST13, AAG2, FAM10A1, SNC6	P50502
HOP	HOP	STI1, STIP1	P31948
HSP90 (HSPC)	HSP90A	HSP90AA, HSP86, HSPC1, HSPCA, LAP2	P07900
	HSP90B	HSP90AB, HSP84, HSPC2, HSPCB,	P08238
CHIP	CHIP	STUB1	Q9UNE7

Table S4: List of primers

PRIMER NAME	SEQUENCE 5'-3'	FORWARD/REVERSE	USED FOR
ecoRV xhoI SOD for	CAGTTCGATATCGCTCGAGCTGCGACGAAG GCCGTGTGCGTGCTG	for	mCherry-SOD1
SOD bamHI notI rev	CGGACGCGGCCGCGGATCCTTATTGGGCGA TCCCAATTACACC	rev	mCherry-SOD1
for hind-cherry	CGTCCAAGCTTATGGTGAGCAAGGGCGAGG AG	for	mCherry-SOD1
rev cherry-ecoRV	GCACTGATATCCTTGTACAGCTCGTCCATGC	rev	mCherry-SOD1
for mut sod a4v	GCGACGAAGGTCGTGTGCGTGCTGAAG	for	SOD1-A4V
rev mut sod a4v	CTTCAGCACGCACACGACCTTCGTCGC	rev	SOD1-A4V
HSPA1A/L destroy sapI (5126 A1A and 5159 A1L) for	GTATTGGGCGCACTTCCGCTTC	for	HSPA1A-A1L NBD swaps
HSPA1A/L destroy sapI (5126 A1A and 5159 A1L) rev	GAAGCGGAAGTGCGCCCAATAC	rev	HSPA1A-A1L NBD swaps
HSPA1A destroy xmaI (2895) for	GTGCCGGTGGTCCCGGCCCTGG	for	HSPA1A-A1L NBD swaps
HSPA1A destroy xmaI (2895) rev	CCAGGGCCGGGACCACCGGCAC	rev	HSPA1A-A1L NBD swaps
HSPA1A destroy xmaI (2354) for	GACAACCAACCCGGCGTGCTGATC	for	HSPA1A-A1L NBD swaps
HSPA1A destroy xmaI (2354) rev	GATCAGCACGCCGGGTTGGTTGTC	rev	HSPA1A-A1L NBD swaps
HSPA1L create sapI (2732) for	CATGAAGAGCGTTGTGAGTG	for	HSPA1A-A1L NBD swaps
HSPA1L create sapI (2732) rev	CACTCACAACGCTCTTCATG	rev	HSPA1A-A1L NBD swaps
HSPA1L create xmaI (2931) for	GAGGATGCCCCGGGCCTGCCTGCGGAAC	for	HSPA1A-A1L NBD swaps
HSPA1L create xmaI (2931) rev	GTTCCGCAGGCAGGCCCGGGGCATCCTC	rev	HSPA1A-A1L NBD swaps
HSPA1L destroy xmaI (2393) for	CTGACAACCAACCCGGCGTGCTGATC	for	HSPA1A-A1L NBD swaps
HSPA1L destroy xmaI (2393) rev	GATCAGCACGCCGGGTTGGTTGTCAG	rev	HSPA1A-A1L NBD swaps
sapI destroy hspa1a/l for	GTATTGGGCGCACTTCCGCTTC	for	HSPA1A-A1L CTD swaps
sapI destroy hspa1a/l rev	GAAGCGGAAGTGCGCCCAATAC	rev	HSPA1A-A1L CTD swaps
sapI create HSPA1L for	CATGAAGAGCGTTGTGAGTG	for	HSPA1A-A1L CTD swaps
sapI create HSPA1L rev	CACTCACAACGCTCTTCATG	rev	HSPA1A-A1L CTD swaps
HSPA1A-E175Q MUT REV	GATGGCGGCGGCCGTGGGCTGGTTGATGAT CCGCAG	rev	HspA1A ATPase mutants
HSPA1A-E175Q MUT FOR	CTGCGGATCATCAACCAGCCCACGGCCGCC GCCATC	for	HspA1A ATPase mutants
HSPA1A+HindIII FOR	CAAGGGGGAGACCAAAGCTTTCTACCCCGA GGAGATCTCG	for	HspA1A 1-111L swaps
HSPA1A+HindIII REV	CGAGATCTCCTCGGGGTAGAAAGCTTTGGTC TCCCCCTTG	rev	HspA1A 1-111L swaps
HspA1L-E177Q For	GCTAAGAATCATCAATCAGCCCACGGCTGC TGCCATTGCC	for	HspA1A ATPase mutants
HspA1L-E177Q Rev	GGCAATGGCAGCAGCCGTGGGCTGATTGAT GATTCTTAGC	rev	HspA1A ATPase mutants
V640 to CCPGCC For	TATGAGAAGTTTTGTTGTCCAGGTTGTTGTA GTGAAGATGATCGTAAC	for	HSPH2-FLASH
V640 to CCPGCC Rev	ATCATCTTCACTACAACAACCTGGACAACA AAACTTCTCATATTCACC	rev	HSPH2-FLASH